HEMOPHILIC BACILLI—THEIR MORPHOLOGY AND RELATION TO RESPIRATORY PIGMENTS.*†

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INTRODUCTION.

PFEIFFER in his classical work[1] upon influenza in 1893 gave a very clear and comprehensive report on the biology of the bacillus that he found associated with the disease which at that time was pandemic. He also found in a few cases of broncho-pneumonia a bacillus somewhat larger than the influenza bacillus and with a marked tendency to form threads; in other respects it closely resembled the influenza bacillus, and he therefore called it the pseudo-influenza bacillus. The most characteristic property of these bacilli is that they require for their growth the presence of hemoglobin and therefore have been frequently called, especially by the French, hemophilic bacteria. Since Pfeiffer's work organisms of a similar character have been found in the throats in a large variety of diseases, especially those of an infectious character, and also occasionally in normal throats.[2] By many they have been considered identical with the influenza bacillus, and the cases in which they were found have been looked upon as double infections, the influenza bacilli being secondary to some other invader. By some observers certain hemophilic bacteria are regarded as the cause of whooping-cough because of their occurrence in the sputum in this disease, but definite proof of their etiologic rôle is still lacking.

It is the purpose of this paper to compare hemophilic bacilli obtained from various clinical conditions with one another, especially from the standpoint of morphology and cultural reactions, not only toward hemoglobin and its modifications but also toward other respiratory proteids which occur in lower animals.

*Received for publication October 30, 1906.

†This work was made possible by a fund given by Mrs. F. R. Lillie for the study of whooping-cough.

[1] *Ztschr. f. Hyg.*, 1893, 13, p. 357.

[2] Davis, "The Bacteriology of Whooping-Cough," *Jour. Infect. Dis.*, 1906, 3, p. 1.

Hemophilic bacilli were obtained chiefly from the sputum, from a variety of conditions including measles, whooping-cough, varicella, bronchitis, cerebro-spinal meningitis, tonsilitis, clinical influenza (grippe), otitis media following measles, and normal throats. Over 100 strains of the bacilli were isolated in pure culture and their morphology and cultural properties noted. The means of isolation was the blood-agar plate. The sputum is washed in water or broth and tubes of melted agar, to which a few drops of blood (preferably pigeon) has been added, are inoculated with washed sputum, and poured into sterile plates. After 24 and 48 hours the plates are carefully examined with the naked eye and also with a hand lens or low-power microscope for the minute dew-drop-like colonies characteristic of hemophilic bacteria, which now are transferred to blood-agar tubes and studied further.

MORPHOLOGIC CHARACTERISTICS.

In smears made from the sputum directly the bacilli are always single with practically no tendency to form threads; neither are they arranged end-to-end in chain formation. In some particles of sputum they are much more numerous than in others but a characteristic grouping does not occur. The arrangement of the bacilli in longitudinal rows or like a school of fish, frequently observed in smears of sputum, is purely an artefact due to the smearing, and not, as some have thought, a characteristic and natural distribution of the bacilli in the mucus. The bacilli are small rods with rounded ends about two or three times as long as broad. They are non-motile, do not have a capsule, do not form spores and do not take Gram's stain. With methylene blue they stain more deeply at the ends; with carbol-fuchsin they stain very distinctly and quite uniformly. In cultures there is more variability in the morphology of the bacilli. Many strains possess a remarkable tendency to form threads though showing none of this in the sputum. All the strains in cultures show it to some extent, but vary considerably in this respect. This tendency often shows itself in the first generation on the plates and in the first 24 hours. Not only do strains, isolated from various conditions, show marked variation, but the same strain at different times may vary. On the whole it may be said that the

more rapidly and the more luxuriantly the bacilli grow the less is this tendency, while the more unfavorable the media the more thread-forms appear; but if these latter are again grown under more favorable conditions they revert to their original form. As a rule, too, the thread-forms are more numerous in a culture a few days old than in a fresh one. The threads are occasionally very long, sometimes crossing the entire field of the microscope, and are often curved in various shapes. They are much wider than the single typical bacillus and usually stain well and quite uniformly.

In view of the fact that these bacilli have such a tendency to produce anomalous forms on ordinary media, and especially under slightly unfavorable conditions, the growth of seven strains was tested upon blood agar containing sodium chloride in varying concentrations. Upon media with a sodium-chloride content of 2 per cent to 3 per cent unusual forms are encountered invariably. In 24 hours at 37° C. only a few peculiar forms are noted; but in 48 hours a great variety of them appears. Swollen forms which assume various shapes are especially numerous. Sometimes the whole bacillus is transformed into a large, round, irregularly staining coccus. Again, one or both ends of the bacillus may be greatly swollen and frequently the ends stain deeply with methylene blue, often resembling very closely certain forms of diphtheria bacilli, the deeply staining parts looking much like granules. While some thread-forms appear they do not occur in great numbers nor to the extent that they do at times on ordinary blood agar. The strains differ from one another considerably in their appearance under these conditions and the same strain may vary considerably at different times.

Hankin and Leumann[1] called attention to the occurrence of degeneration and involution forms of the pest bacillus when grown on NaCl agar and suggested this as a means of separating this organism from other organisms having a similar appearance. Since then it has been shown by several, especially by Matzuschita[2] and Rosenfeld[3] that many other varieties of bacteria display this phenomenon on NaCl agar after 48 hours or later, but that the forms of the pest

[1] *Centralbl. f. Bakt.*, 1897, 22, p. 438.

[2] *Ztschr. f. Hyg.*, 1900, 35, p. 495.

[3] *Centralbl. f. Bakt.*, 1901, 30, p. 641.

bacillus occur in 24 hours and are sufficiently characteristic to be of value in its differentiation. The influenza bacilli, therefore, behave on sodium-chloride media like many other forms of bacteria, but there is nothing characteristic in these changes and they are therefore of no service in distinguishing these bacilli from other forms; neither do the various strains show any characteristic appearances which might indicate differences between them.

CULTURE OF HEMOPHILIC BACILLI.

In the cultivation on hemoglobin agar of numerous strains of hemophilic bacilli from the various clinical conditions already noted no differences were seen. On plates they appear as small, non-hemolyzing, clear, dew-drop-like colonies, as a rule, pin-point in size, and often requiring the aid of a hand lens for detection. By reflected light they appear a delicate, pale blue. Under a low power they are quite homogeneous, circular or oval in shape, have a regular margin and no central nucleus. On blood-agar tubes a delicate growth occurs along the needle track, often difficult to see at first glance. The colonies tend to remain discrete.

Hemoglobin seems to be a necessary substance for the continued growth of the bacteria. Pfeiffer first showed this for the influenza bacillus, tests on many other kinds of media aways giving negative results. Cantani[1] says that he obtained growth of influenza bacilli upon spermatic fluid, and many other substances free from hemoglobin, and thinks that globulin may be the active part of hemoglobin, which induces their growth; but Ghon and Preyss[2] showed that the media he used were not entirely free from hemoglobin or hematin. Fichtner[3] also contends that hemoglobin is not necessary, and thinks there is some substance in the red cells which is essential for multiplication and explains certain symbiotic phenomena in this way. He says he succeded in getting a good growth on agar if sputum, heated to 60–65° C. for several days until sterile, was mixed with it. Notwithstanding these and a few other conflicting results, most observers are agreed that hemoglobin is the essential substance and that no

[1] *Ztschr. f. Hyg.*, 1901, 36, p. 29.

[2] *Centralbl. f. Bakt.*, 1904, Orig., 17, p. 531.

[3] *Ibid.*, Orig., 35, p. 374.

other substance has yet been found to take its place successfully in the cultivation of influenza bacilli.

Pfeiffer used the blood of various animals, including man, rabbit, guinea-pig, pigeon, and fishes, and found pigeon blood to be the most successful of all the bloods tested for this purpose. He thinks this is due to the instability of the pigeon corpuscles which allows the hemoglobin to diffuse more readily into the media. Whether this latter statement is true or not, it does appear to be a fact, as many have determined that pigeon blood is a very suitable medium, and, as a rule, more luxuriant growth is obtained on this than with many other bloods. I have used pigeon blood very largely in the isolation of the bacilli and it seems more reliable than other bloods and especially more so than human blood.

Because of the value of pigeon blood in the cultivation of hemophilic bacilli a convenient method of obtaining it in large quantities in a sterile condition becomes important. In 1902 Czaplewski[1] described a method of making an incision into the large breast muscle of the pigeon with a sharp lance; as the blood oozes from this wound, it is drawn into a small pipette and introduced into melted agar. Another method frequently used is that of pricking a vein in the wing of the bird and allowing the blood to drop into tubes of media or upon agar plates. Evidently these methods are unsuitable when a large amount of pigeon blood is desired and when defibrinated blood or a quantity of serum is wanted. A more convenient and rapid method, certainly less liable to result in contamination, is one which I have used, the details of which are as follows: The feathers are removed on the left side of the pigeon directly underneath the wing, exposing the skin for several square centimeters. The skin is then thoroughly cleansed with 5 per cent carbolic acid and alcohol. The pigeon lying on its right side, an assistant holds up the left wing and extends the left leg. At a point approximately midway between the posterior skin fold of the wing and a fold of the skin found just anterior to the thigh when the bird is in this position, the needle of the Luer syringe is introduced directly into the heart. The needle passes just above the margin of the breast bone and proceeds, approximately vertical to the surface, inward about 1.5 to 2 cm., when the beat of the heart may be distinctly felt against the needle. The heart beat is important and should always be obtained before proceeding further. Advancing the needle a short distance the blood will be seen to enter the syringe. The needle should be sharp so as to enter the heart readily, of medium size, and from 3 to 4 cm. in length. Large needles do not enter the heart readily and are apt to push it aside, while small needles allow the blood to pass too slowly into the syringe, furnishing an opportunity for clotting. By dissecting a dead pigeon one can readily determine the proper position at which to enter the heart.

Four c.c. may be removed without any evil effects. If more is taken the animal usually shows weakness of the legs and other signs, but frequently recovers if 7 or 8 c.c. are withdrawn. The needle is removed cautiously and practically no bleeding occurs

[1] *Centralbl. f. Bakt.*, 1902, 32, p. 667.

at the site of the puncture. The blood is at once transferred to a sterile tube and defibrinated with a wire. This usually takes from two to four minutes. Four c.c. can be withdrawn from the same bird once every week or 10 days and produce no ill effects. I have one pigeon which has been bled from the heart about 25 times in six months and has furnished over 100 c.c. of blood in that time, yet it is as fat and plump as when first bled. After the fibrin is removed the blood can be kept in a capped test-tube in the ice-box for several weeks. For plating about six drops are introduced into melted agar cooled to 43° C., which is then inoculated and poured into the Petri dish. For agar slants one or two drops allowed to run over the surface are amply sufficient. Or a solution of hemoglobin may be obtained by adding distilled water to the blood, thus increasing its volume several times. Four or five drops of this solution suffice to cover the agar slant, or the blood or hemoglobin may be added directly to melted agar, which is then slanted. Such tubes are very convenient, as the influenza colonies are easily seen on the surface, and in transferring an organism to non-hemoglobin media for the purpose of testing its growth there is much less danger of removing hemoglobin with the organisms on the platinum loop.

Careful examination of the propreties of hemoglobin derived from various animals shows that they are not all identical, the hemoglobins varying in chemical composition, in solubility, in crystalline form, and in the quantity of water of crystallization, but apparently all perform the physiological function, of transmitting oxygen. It is therefore important to test the blood of a number of both warm and cold-blooded animals to see if the differences in the hemoglobin have any effect upon the growth of the bacilli. The blood, obtained in sterile condition, was added to plain agar both by smearing on the surface of slants and also by mixing it thoroughly with melted agar and then slanting the tube. The results are given in Table 1.

TABLE 1.

GROWTH OF HEMOPHILIC BACILLI IN MEDIA CONTAINING BLOOD OF VARIOUS ANIMALS.

	Growth of Bacilli	Respiratory Proteid in Blood
Mammals	+	Hemoglobin
Birds	+	"
Perch	+	"
Eel (*Anguilla chrysypa*)	+	"
Dog-fish (*Mustelus canis*)	+	"
Snapping turtle (*Chelydra serpentina*)	+	"
Painted turtle (*Chrysemys picta*)	+	"
Frog	+	"
King crab (*Limulus polyphemus*)	o	Hemocyanin
Lobster (*Homarus americanus*)	o	"
Spider crab (*Libinia dubia*)	o	"
Clam (*Mya arenaria*)	o	"
Phascolosoma Gouldii	o	Hemerythrin
Nereis virens	?	Hemoglobin
Sea-cucumber (*Thyone briareus*)	o	"
Star-fish (*Asterias vulgaris*)	o	"
Sea-urchin (*Arbacia punctulata*)	o	Echinochrom

Inasmuch as hemoglobin belongs to an interesting class of substances known as respiratory proteids, it is important that the growth of influenza bacilli be tested with other compounds of this class, especially hemocyanin, a common proteid in many of the lower animals. This substance apparently performs the same physiological function as hemoglobin but contains copper in large amounts and no iron. The results with the blood of these lower forms are also given in Table 1. Some of the bloods tested were from marine animals* in many of which the salt content is about the same as that of sea-water (3–4 per cent). The blood, however, was diluted to such an extent in the media that the salt content could have no special effect on the growth of the bacilli which grow on media containing as high as 3 per cent sodium chloride.

In Table 1 "mammals" include the following animals: man, dog, rabbit, guinea-pig, ox, sheep, and horse. Among the birds blood from the pigeon and hen were tested. The bloods containing hemocyanin were examined for hemoglobin with a spectroscope so as to exclude the possibility of this substance being present. With the blood of the sea-cucumber (*Thyone briareus*) the spectrum of hemoglobin is distinctly obtained. In its blood are seen large, round, nucleated cells, staining deeply with eosin, which contain the pigment. It is difficult to get the blood in appreciable quantities and sterile. I succeeded twice in getting small quantities in a sterile condition, but in neither case did the influenza bacilli grow in media made with this blood. This is the only instance I have found in which the growth of hemophilic bacilli was not obtained in the presence of hemoglobin. If one considers the echinoderms as being below the annelids in the zoölogical scale, as is commonly done, then the sea-cucumber is the lowest animal in which hemoglobin occurs. From *Nereis*, an annelid in which the hemoglobin is dissolved in the plasma, I was never able to get the blood in an absolutely sterile condition. The bacilli grew upon this blood, but since there were a few other organisms present, symbiosis probably influenced the result. By heating the blood for several days at 65° C it was rendered sterile, and upon this heated blood the bacilli did not grow. After such

* This part of the work was done at the Marine Biological Laboratory at Woods Hole during the summer of 1906.

prolonged heating, however, all the hemoglobin was probably changed to hematin, so that this negative result is of no value.

The body fluid of *Phascolosoma*, another annelid, according to Schwalbe[1] contains a respiratory pigment which is called hemerythrin. It does not give a characteristic spectrum, contains iron, and unites more firmly with oxygen than hemoglobin. This fluid is easily obtained in large amounts in a sterile condition. Many attempts to grow hemophilic bacilli on media to which this fluid was added always gave negative results.

Hemocyanin is readily obtained in a sterile condition from a number of the lower animals. Hemocyanin agar was made containing varying quantities of hemocyanin, both unheated and heated (60° C. for 30 minutes); in no instance did growth of the bacilli occur. When staphylococci were streaked on hemocyanin agar after inoculating with influenza bacilli the latter grew no better than upon plain agar when so streaked (influenza bacilli often grow, at least through a few generations, in conjunction with another organism in the absence of hemoglobin). Hemophylic bacilli do not grow on media containing echinochrom, an iron-containing pigment from the sea-urchin.

From Table 1 we may conclude then, that hemophilic bacilli can use the hemoglobins from a large variety of animals, both warm and cold-blooded, fresh and salt-water forms. The differences between the hemoglobins in their chemical and physical properties do not appear to have any effect upon their growth.

Hemocyanin, hemerythrin, and echinochrom cannot be used by the bacilli even though they appear to have the same function, i. e., are oxygen-carriers, as hemoglobin.

Cantani and Fichtner, as above stated, maintain that certain substances in the cells other than hemoglobin are suitable for the growth of influenza bacilli. In animals, like Limulus, whose blood contains hemocyanin but no hemoglobin, there is an abundance of white cells, but these appear to be quite unsuitable for the growth of the bacilli; but when a little hemoglobin is added growth occurs at once. It does not appear that there is any substance in the blood of animals, containing hemocyanin, which prevents growth when

[1] *Arch. f. mikroskop. Anat.*, 1896, 5, p. 248.

hemoglobin is present, and it consequently does not seem probable, as Cantani contends, that cell substances, such as forms of lecithin, cholesterin, or other bodies, are able to induce growth of the bacilli.

In the cultivation of hemophilic bacilli upon various special media not containing hemoglobin I have had no success. Yolk of egg smeared on or mixed with agar gives negative results. The hematogen, therefore, of Bunge, an iron compound, supposed to be the prosubstance of hemoglobin in the egg, does not appear to be useful for the growth of these organisms. Sputum, filtered through clay or rendered sterile by heating at 65° for several days and mixed with agar, likewise gave negative results. Bile obtained with care to avoid the presence of hemoglobin gave negative results.

In order to determine, if possible, whether the nutrient property of hemoglobin is dependent upon its power of giving up oxygen readily, chemical substances which give up oxygen easily were added in small quantities to the media, but growth of the bacilli did not take place on media containing sodium nitrate, sodium nitrite, potassium chlorate, collodial platinum, hydrogen peroxide, and collodial platinum plus hydrogen peroxide. When collodial platinum and hydrogen peroxide together are added to melted agar, the medium in 24 hours is filled with small bubbles of liberated oxygen. Under these conditions, however, no growth was obtained. Iron salts of various kinds in small amounts were added to media, but in no instance was growth of influenza bacilli detectable; this agrees with the results obtained by Pfeiffer and others.

Hematin, the iron-containing moiety of hemoglobin, according to several observers will not support the growth of influenza bacilli; according to Ghon and Preyss[1] it will do so if another organism (staphylococcus) is present. Since they will grow for several generations when mixed with another organism on media which is spectroscopically free from hematin, the latter observation is of little significance. Pure hematin dissolved in dilute alkali and added to media in varying proportions gave uniformly negative results. The experiment was properly controlled to rule out any possible inhibiting effect of the alkali upon the growth of the bacilli.

[1] *Centralbl. f. Bakt.*, 1904, Orig., 17, p. 531.

Experiments were made to determine the effect of heated hemoglobin on the growth of hemophilic bacilli. At a temperature of about 65° C., or slightly less, a solution of hemoglobin is coagulated. In media containing solutions of hemoglobin heated for 30 minutes or one hour at this temperature the influenza bacilli will grow. If the heated solution is smeared on the surface of agar slants, the influenza colonies cluster about the clumps of coagulated hemoglobin. The bacilli will even grow in the presence of hemoglobin heated to boiling for a few minutes. But if the solution is heated to 65° C. for several days, or in an autoclave at 110° C. for 30 to 60 minutes, then the bacilli no longer can use it. Now at a temperature of 60° C. or less oxyhemoglobin is slowly split into its constituents, hematin and an albuminous portion, and in view of this fact the results just noted are readily explained. Heating to 65° C., or even to 100° C., for a short time is not sufficient to split all the oxyhemoglobin. But by heating for a long period at a temperature of 60°–65° C., or at a higher temperature in an autoclave for an hour, the hemoglobin is split entirely into its constituents and then is no longer serviceable for the bacteria. One cannot directly follow the transformation of hemoglobin with a spectroscope because coagulated hemoglobin does not give a spectrum.* If, to an aqueous solution of oxyhemoglobin, a small amount of sodium carbonate is added and then heated to 55° C., the solution in a short time becomes deep-brown in color but no coagulation occurs, even when heated to boiling. The spectroscope shows that the hemoglobin spectrum is replaced by that of hematin. In such a solution influenza organisms will not grow, showing again that alkaline hematin cannot be used by these bacteria.

We may therefore conclude from these experiments that hemophilic bacilli can use hemoglobin and coagulated hemoglobin in their development, but as the hemoglobin is split into its constituents by heating it loses its power to support their growth.

* Since the spectroscopic test cannot be applied to coagulated hemoglobin the question may be raised whether or not coagulation of hemoglobin is not merely a decomposition into hematin and a globulin since the splitting of hemoglobin into hematin and an albuminous portion begins below the temperature of coagulation. The fact that influenza bacilli cannot grow on hematin and can grow well in the presence of coagutaled hemoglobin may be used as evidence that this process of coagulation of hemoglobin is not, at least altogether. a transformation of hemoglobin into hematin and a globulin.

AMOUNT OF HEMOGLOBIN NECESSARY FOR THE GROWTH OF HEMOPHILIC BACILLI.

In order, if possible, to throw some light upon the rôle that hemoglobin plays in the development of hemophilic bacteria quantitative experiments were made to determine how much hemoglobin in solid media is necessary.

Human blood was diluted in distilled water 1,000 times, and this solution was added to measured quantities of melted agar at a temperature of 42° to 45° C. In this way various dilutions of the blood were made and the growth of the bacilli tested with them. Assuming that hemoglobin is the essential element in the blood for the growth of these organisms and knowing the amount of hemoglobin in blood we can easily calculate the amount of hemoglobin in the various dilutions. Preyer[1] has determined that in human blood hemoglobin makes up approximately 14 per cent or one-seventh of the weight of the blood. Therefore by multiplying the dilution of blood by seven we get approximately* the dilution of the hemoglobin.

In transferring from the stock culture it is necessary to keep in mind that some of the hemoglobin may adhere to the bacteria and the needle; in order to eliminate this source of error transfers must be made through several generations. A typical hemophilic organism isolated from a case of whooping-cough was transferred from a diffuse blood-agar culture, care being taken just to touch the surface of the growth so as to transfer as little as possible of the hemoglobin. In this first series growth occurred at a dilution of one part of hemoglobin to 180,000 parts of media. The controls on plain agar showed no growth. Transfers were now made from the first series of tubes to corresponding dilutions in a second series, and so on through five generations. In the second generation the highest dilution at which growth occurred was 1:60,000; in the third generation, 1:90,000; in the fourth, 1:180,000; and in the fifth, 1:60,000. Careful controls were made on the same media as that to which the hemoglobin had been added. Similar experiments made with pigeon blood gave essentially the same results. This would seem to indicate

[1] *Am. Text Book of Phys.*, 1, p. 38.

* The hemoglobin content of blood from different animals and from the same animal varies somewhat, so that the figures are only approximate. For our purpose, however, it is necessary only to obtain approximate dilutions.

that pigeon blood is no more favorable for the growth of the bacilli than other blood at least in a laked condition and in high dilutions. With dog blood the highest dilution at which growth occurred was 1:90,000.

The hemoglobin solutions in high dilutions deteriorate rapidly, so that it is necessary to use fresh solutions each day. A solution of 1:10,000 in a few days changes its color from a delicate pink to a light yellow, and with such solutions the bacilli grow only at much lower dilutions.

The results show that these bacilli are very sensitive to small amounts of hemoglobin, and this raises the question as to how hemoglobin favors the growth of the organisms. In the extremely high dilutions it does not seem that the hemoglobin can be of much importance as a nutritive factor. It is more probable that it plays the part of a ferment in some essential process, perhaps that of respiration. While the process may in some way be dependent upon the iron, owing to its peculiar combining relationships with oxygen in the hemoglobin molecule, the iron in itself cannot be said to be more essential than any other element, because every compound of iron tested was of no value in cultivation of the bacilli. Oxidases are not the determining factor, for the blood of lower forms contains them, yet without hemoglobin they are of no avail. One naturally associates the function of hemoglobin as an oxygen-carrier with its function of causing hemophilic bacteria to grow. But hemocyanin, a copper compound, apparently performs the same function as an oxygen-carrier in many of the lower animals, but the bacilli cannot use it. The copper in this form does not appear to be fatal to them, since, as we have seen above, they will grow in media containing hemocyanin if hemoglobin is present. Hemerythrin, occurring in *Phascolosoma*, is an iron-containing compound similar to hemoglobin, but it does not permit growth. According to Luerssen[1] chlorophyll, a substance closely related to hemoglobin in some respects but containing no iron, does not favor the growth of influenza bacilli. The facts at present therefore permit us to state that hemophilic bacteria will grow only in the presence of hemoglobin and very minute amounts of this body suffice for this purpose. This

[1] *Centralbl. f. Bakt.*, 1904, 35, p. 437.

suggests that the hemoglobin acts as a catalytic agent rather than as a nutritive substance.

In connection with these facts should be mentioned the favoring influence that growing bacteria of another variety have upon hemophilic organisms. My own experiments indicate that continuous growth of hemophilic bacteria will not take place in the presence of other bacteria, but that they die out after a few transfers have been made. Some observers, especially Neisser,[1] claim to have succeeded in carrying them in mixed cultures through many generations. However this may be, it is certain that other living organisms distinctly favor the growth of hemophilic bacteria, and the most luxuriant growth is obtained by a combination of hemoglobin and the presence of another organism, as is done, for instance, by inoculating the whole surface of a blood-agar tube with influenza bacilli and then making a staphylococcus streak through the center. It would be reasonable to suppose that there is something in common in the way in which these two favoring influences operate, but on this point we can only speculate.

OTHER PROPERTIES OF HEMOPHILIC BACTERIA.

In a number of strains of hemophilic bacteria grown for about one year and transferred every 10 to 14 days, the properties and morphology have remained practically unaltered.

In fluid media they live longer as a rule than in solid media, which is probably due to the effect of drying. On solid media in uncapped tubes I have several times obtained growth after an interval of one month. In sealed glass tubes kept at room temperature in the dark the bacilli grew after three months. In another tube similarly kept and examined after eight months, the bacilli showed no growth upon the inoculation of fresh media.

In distilled water the bacilli gave good growth at the end of 24 hours, scanty growth at the end of 48 hours, and none at all after 72 hours. Dried on a free surface kept sterile the bacilli gave good growth after three hours, slight growth after 24 hours, and none after 48 hours. In human defibrinated blood the bacilli at the end of 10 days were alive and present in abundance. In human serum

[1] *Deutsche med. Wchnschr.*, 1903, 29, p. 462.

no growth was obtained after 24 hours. The organisms, then, are not highly resistant, dying rapidly in water and being quickly killed by drying; in defibrinated blood, however, they find a favorable medium for development and remain alive for a long period.

They grow best at 36°–38° C., and do not grow at room temperature. Growth is first detected at a temperature of about 28° C. A temperature of 42°–43° C. for a few hours is fatal.

The organisms after growing on artificial media containing small amounts of hemoglobin (one drop of blood and frequently less to 5 c.c. of melted agar) for many months do not seem to lose their hemophilic property to any perceptible degree, for they show not the least evidence of multiplication when transferred to non-hemoglobin media. It is possible that by much longer cultivation and on media containing much less hemoglobin they may adapt themselves to plain media, but no evidence for this has thus far been obtained.

CONCLUSIONS.

1. Various strains of hemophilic bacteria isolated from different conditions cannot be differentiated on morphologic grounds.

2. They can utilize in their growth hemoglobins from various warm and cold-blooded animals and also from fresh-water and marine forms.

3. They are not able to utilize other respiratory proteids, e. g., hemocyanin, hemerythrin, echinochrom.

4. An extremely small amount of hemoglobin in the media (1 part in 180,000 parts of media) is sufficient for their development.

5. No satisfactory cultural results have been obtained with any substance other than hemoglobin. They do not grow in the presence of hematin.